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BREEDING OF A TOMATO GENOTYPE READILY ACCESSIBLE TO GENETIC MANIPULATION

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A tomato genotype, superior in regenerating plants from cell cultures, was obtained by transferring regeneration capacity from *Lycopersicon peruvianum* into *L. esculentum* by classical breeding. This genotype, MsK93, greatly facilitates genetic manipulation of tomato, as was demonstrated by successful leaf disc transformation using *Agrobacterium tumefaciens* and by direct gene transer to protoplasts derived from callus.

Key words: Lycopersicon esculentum; Lycopersicon peruvianum; tomato; *Agrobacterium tumefaciens;* kanamycin resistance; regeneration; transformation

Introduction

Tomato (*L. esculentum* Mill.) is an important crop species of which many physiological and genetical aspects have been studied. Successful application of molecular and cellular biological techniques have been hampered, however, by the rather unfavourable cell culture and regeneration characteristics of the cultivated tomato. As compared with *L. esculentum*, the related species *L. peruvianum* (L.) Mill. is superior with respect to plant regeneration from cell and protoplast cultures [1–3]. Therefore transfer of the regeneration trait from the wild species to the cultivated tomato has been suggested [3]. A major problem in this approach, however, lies in hybridizing these species, because when *L. esculentum* is used as male parent, pollen tube elongation is arrested before fertilization, whereas embryo abortion occurs when *L. esculentum* is the female parent [4]. This report describes the breeding of a tomato genotype that combines regeneration characteristics of *L. peruvianum* and crossability with *L. esculentum*. The application of this genotype in genetic manipulation was demonstrated by its successful transformation using both *A. tumefaciens* and direct gene transfer to callus derived protoplasts.

Abbreviations: ATW, Agrobacterium Tomato Wageningen; BA, benzylaminopurine; MS, Murashige-Skoog; NAA, naphthaleneacetic acid; SDS, sodium dodecyl sulfate; PEG, polyethylene glycol.

Materials and methods

Plant material

An F_3 hybrid population of *L. peruvianum* × *L. esculentum* (IVT 741505) was donated by Dr. Hogenboom (Institute for Horticultural Plant Breeding (IVT), Wageningen). This population was derived from two rare successful crosses which involved a *L. peruvianum* genotype selected for reduced *L. esculentum* pollen tube inhibition [5] (Fig. 1). The male sterile mutant, *ms-10*^{35} [6] of *L. esculentum* (VF 11 background) was a gift from Professor Rick at Davis, CA, U.S.A.

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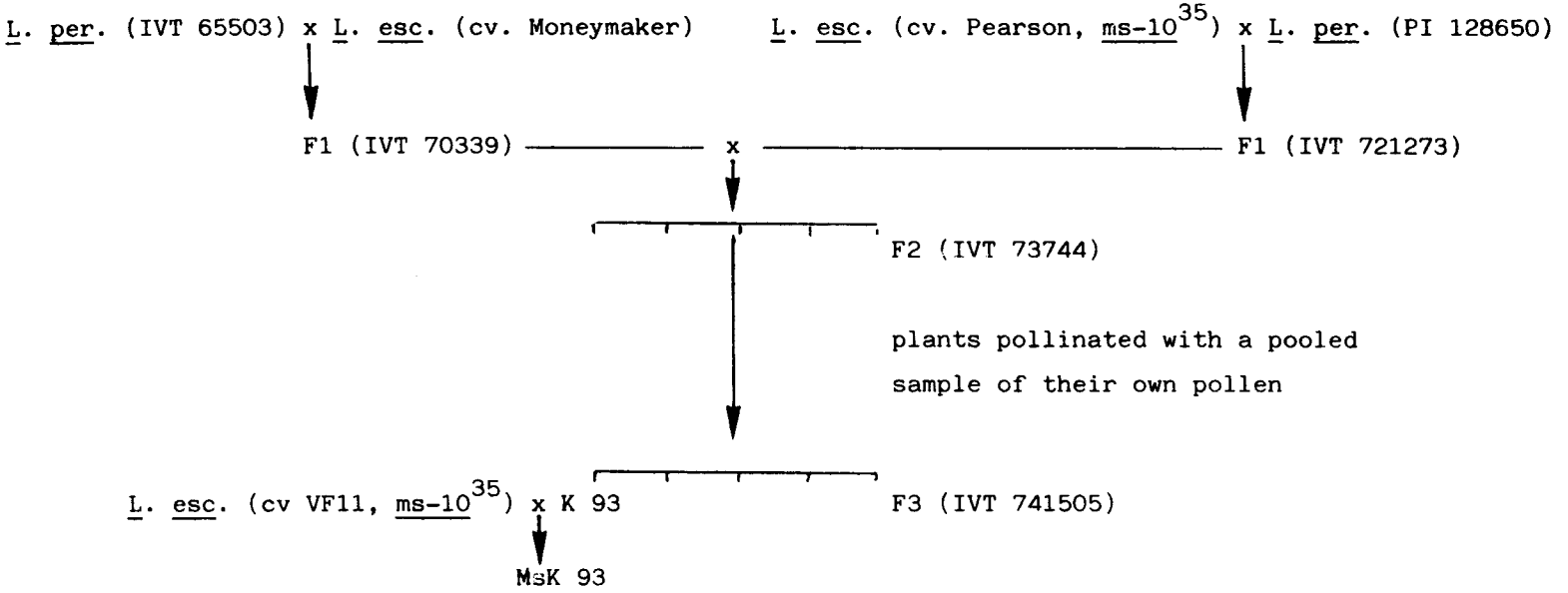

Fig. 1. Pedigree of MsK93. *L. per.*, *Lycopersicon peruvianum*; *L. esc.*, *Lycopersicon esculentum*; cv, cultivar; IVT no., seed number of the Institute of Horticultural Plant Breeding (IVT); PI no., Plant Introduction number, *ms-10*^{35}, allele 35 of the *ms-10*, male sterility, gene.

Bacterial strains and plasmids

The *A. tumefaciens* strain C58 (pGV 3850 :: 1103 neo dim) was obtained from Dr. M. van Montagu, Ghent, Belgium and has been described by Czernilofsky et al. [7].

For direct gene transfer experiments plasmids pneoΔ18 and pCTW19, 22 and 78 were used. Plasmid pneoΔ18 [8] was obtained from Dr. Bevan, PBI, Cambridge, U.K. and is a pUC9 plasmid with a chimaeric kanamycin resistance gene (the neomycin phosphotransferase II gene from Tn5 under transcriptional control of the regulatory elements of the nopaline synthase gene from *A. tumefaciens).* The plasmids pCTW19, 22 and 78 are derivatives of pneoΔ18 in which various tomato DNA sequences preselected as described by Zabel et al. [9] were cloned at the HindIII site (pCTW78) or EcoRI site (pCTW19 and 22).

Tomato tissue culture and protoplast techniques

Leaf discs (5 mm) were punched from surface sterilized leaves of young plants grown in a greenhouse and shoots were induced on these explants using 2Z medium [10] which contains 2 mg l^{-1} zeatin. Shoots were then rooted on a Murashige-Skoog (MS) medium without hormones and containing 1% sucrose. Callus was induced (on similar explants) on R3B medium [11], which is an MS based medium containing as hormones 2 mg l^{-1} naphthaleneacetic acid (NAA) and 1 mg l^{-1} benzylaminopurine (BA) and which was used for all callus cultures. Plant regeneration on callus was achieved by (repeated) transfers to 2Z medium [10]. All cultures were grown at 16 h light (approx. 2000 lux) and transferred to new plates at least every 4 weeks.

Callus to be used for the isolation of protoplasts was grown on R3B medium [11] containing only 0.5 mg l^{-1} BA. Two weeks after the last transfer, 1.5—2.0 g of callus was incubated for 3—4 h in an enzyme solution Cellulase RS, 0.5%; Macerozyme 0.25%; Pectolyase Y23 0.05%, dissolved in the protoplast culture medium (ATp) of Adams and Townsend [12] under continuous shaking (30 rev./min) in a waterbath at 30°C. The protoplasts were purified by flotation on 21% sucrose, using low speed centrifugation (5 min, 160 × *g*) and cultured in liquid ATp medium [12]. After 4—6 days, when the first divisions could be observed, the cultures were diluted 1:1 with fresh ATp medium [12] and transferred from the dark to low light conditions (approx. 200 lux). After repeated dilutions with ATp medium containing 5.5% mannitol, microcalli were transferred at about 7 weeks after protoplast isolation to solid ATc medium [12]. From this medium they were transferred to ATs medium [12] and subsequently to 2Z [10] for shoot regeneration.

Leaf disc transformation

Transformation of leaf discs by means of *A. tumefaciens* was performed according to Horsch et al. [13] with some modifications. Leaf discs, prepared as described above, were dipped in an overnight culture of *A. tumefaciens*, blotted dry on sterilized filter paper and placed with their top side up on a feeder-layer of Petunia 'Commanche-albino' suspension cells on 2Z medium [10]. The dishes were sealed with parafilm and incubated for 2 days. Thereafter, the leaf explants were transferred to new 2Z plates [10] containing 200 mg l^{-1} cefotaxime (Calbiochem) and 200 mg l^{-1} vancomycine (Sigma) in order to prevent further bacterial growth. The dishes were then closed with only two small pieces of tape, as a better gas exchange was found to enhance shoot formation and the lowered humidity reduced bacterial growth. At day 11 after leaf disc preparation, the first shoot primordia were visible and the explants were transferred to new plates containing also 100 mg l^{-1} kanamycin (Gibco). For rooting, shoots were transferred to MS medium without hormones and containing 100 mg l^{-1} kanamycin.

Direct gene transfer

Protoplast transformation was performed using the method of Krens et al. [14] including as modifications, a reversal of the order by which DNA and polyethylene glycol (PEG) were added, a shorter incubation with PEG and a more gradual dilution with F medium [14]. To 1 ml of 5×10^5 protoplasts in ATp medium [12], 10 μg plasmid DNA and 50 μg Calf thymus DNA was added and gently mixed with the protoplasts. Thereafter, 0.5 ml of 40% (w/v) PEG 6000, dissolved in F medium was added and the mixture was left for 10 min. Fresh F medium was then added at 10-min intervals (2×1 ml, 2×2 ml and 1×3 ml) followed by pelleting and culturing of the protoplasts as described above. Selection for kanamycin resistance (50 mg l^{-1}) started at about 2 weeks after protoplast isolation.

Cytology

The ploidy level of regenerated plants was determined by counting the number of chloroplasts in guard cells (stained with a 3 g l^{-1} I_2/13 g l^{-1} KI solution), which was checked in a number of cases by counting the number of chromosomes in root tip cells. For the analysis of plants regenerated from protoplasts, only root tip cells were analysed. For chromosome counts, root tips were collected in 8-hydroxyquinolin (350 mg l^{-1}) and after 4 h at 17—20°C the tips were transferred to a 1:3 acetic acid/ethanol mixture, in which they were stored. Roots were then macerated in 1 N HCl for 5 min at 60°C and the chromosomes were stained and squashed in lactopropionic orcein [15].

DNA isolations and Southern blot analysis

Plasmids pneoΔ18, pCTW19, 22 and 78 were isolated using the alkaline extraction procedure described by Birnboim and Doly [16], followed by one CsCl-ethidium bromide gradient banding. Plant DNA was isolated from leaf or callus tissues using the CTAB-procedure of Murray and Thompson [17]. For Southern blot analysis, 4 μg of total plant DNA was digested with 80 units of the restriction endonucleases as specified in the figure legends. Restriction fragments were separated on 1% agarose gels and transferred onto Gene Screen Plus membrane filters employing an alkaline blotting procedure [18]. The probe consisted of a 1.4 kilobase pair Eco RI-SalI fragment containing the nopaline synthase promotor, the kanamycin resistance gene of Tn5 and the nopaline synthase terminator inserted in M13 mp8 [19]. Probes were labelled from an M13 template, using an M13 sequencing primer and the Klenow fragment of DNA polymerase I [20]. Unreacted nucleotides were removed by chromatography on a Sephadex G50 column. The specific activity of the probes was approx. 10^8 cpm/μg. Prehybridization was performed for 24 h at 65°C in 10× Denhardt solution [21], 40 mM Tris—HCl (pH 7.0), 5× SSC, 1 mM EDTA, 1% sodium dodecyl sulphate (SDS) and 100 μg/ml of sonicated denatured salmon sperm DNA. Hybridization was carried out for 40 h at 65°C in fresh prehybridization solution containing about 2 ng/ml of denatured, ^{32}P-labelled DNA probe, prepared as described above. Filters were washed for 10 min in 5× SSC, 1 mM EDTA at room temperature and for 60 min in 1× SSC, 0.1% SDS at 65°C and finally autoradiographed at —80°C using an intensifying screen.

Results

Breeding of the tomato genotype MsK93

From the F_3 population of *L. peruvianum* × *L. esculentum*, 185 plants were tested for their ability to regenerate shoots on established callus cultures. Ultimately, 7 plants remained, callus of which, after being in culture for 1 year still appeared capable of regenerating into plants, upon transfer to 2Z medium [12]. These selected plants were self-fertile and were crossed, on a large scale (hundreds of pollinations), as male parents with several *L. esculentum* geno-

types. Only in one case where plant K93 was crossed with *L. esculentum*, *ms-10*^{35}, a (backcross) hybrid was obtained. This hybrid is hereafter referred to as MsK93. Its pedigree is shown in Fig. 1. The plant is self-fertile and has orange fruits with the size of Cherry-type tomatoes. Although considerable embryo abortion still occurred upon crossing MsK93 as male parent with *L. esculentum* genotypes, gene transfer to the latter species by hybridization was feasible, as about 1—3 hybrids could be obtained from one fruit (normal 50—150 viable seeds). With the next backcross generation, there was virtually no reduction in crossability as compared with crosses within the species *L. esculentum*.

Cell and protoplast culture properties of MsK93

As compared with its *L. esculentum* parent, more shoots were regenerated both on leaf explants and established callus cultures of MsK93. The growth rate of callus of MsK93 was also higher (Table I). The behaviour in cell culture of the *ms-10*^{35} mutant was comparable to most of the *L. esculentum* genotypes that were tested, which included the cultivars Lukullus and VFNT Cherry that were reported [3,11,22] to be some of the best regenerating *esculentum* genotypes. In contrast to *L. esculentum*, callus pieces of MsK93 which did not form shoots after culturing for 8 weeks on 2Z medium (Table I) were capable of regenerating shoots after one or more repeated transfers on this medium. Rooting of shoots in general presented no problems.

Table I. Comparison of tissue culture characteristics of 10 plants of MsK93 (cuttings) and 10 plants of its *L. esculentum* parent (VF11, *ms-10*^{35})

Genotype	Average no. of shoots/ leaf disc[a]	$\frac{W_4-W_0}{W_0}$[b]	Average no. of callus pieces with shoots[c]
ms-10^{35} mutant	3.2 ± 0.7	9.9 ± 0.8	0
MsK93	13.1 ± 2.6	18.8 ± 1.6	6.5 ± 1.0

[a] After 4 weeks on 2Z medium.
[b] W_4 = weight after 4 weeks, W_0 = initial weight of callus after the 4th transfer to fresh R3B medium.
[c] After 2 transfers to 2Z medium following 12 weeks on R3B (3 transfers). The data are based on 10 callus pieces randomly chosen at the moment of the first transfer to 2Z.

Protoplasts from MsK93 isolated from an established callus culture (8 months after induction on explants) produced microcalli with an efficiency of 0.5—2% of the initial number of isolated protoplasts. One hundred and three of such microcalli were chosen at random and tested for their ability to regenerate into plants. At 190 days after protoplast isolation, 91% of these microcalli had formed shoot-like structures, 43% showed normal looking shoots and 17% had produced rooted plants. For 14 of the rooted plants, the chromosomes were counted, showing that 1 had the normal diploid chromosome number ($2n$ = 24), 12 plants were tetraploid ($2n$ = 48) and one plant had a cell with 38 chromosomes together with cells of about twice that number.

Leaf disc transformation

In an experiment using 80 MsK93 leaf discs inoculated with *A. tumefaciens* strain C58 (pGV 3850::1103 neo dim), a total of 73 shoots was obtained and tested on a kanamycin containing rooting medium. Of these, 15 proved to be transformants as judged from their capacity to root on this medium. On the average, rooted transformants were obtained 60 days after leaf disc preparation (range 42—75 days). All of these, 15 transformants were diploid, comparable in morphology to MsK93 and fully fertile except one plant. The plants were named ATW (Agrobacterium Tomato Wageningen) and numbered consecutively. Genetic transformation of the ATW plants was confirmed both by Southern blot analysis, showing the presence of a functional kanamycin resistance gene (Fig. 2) and by the segregation of their

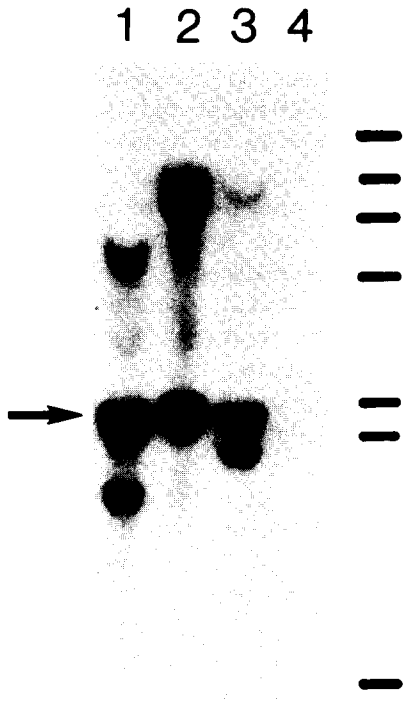

Fig. 2. Southern blot analysis of an EcoRI/SalI double digest of DNA isolated from 3 MsK93 plants separately transformed by *A. tumefaciens* carrying pGV 3850 :: 1103 neo dim. Lane 1, plant ATW7; lane 2, plant ATW13; lane 3, plant ATW15; lane 4, control untransformed MsK93. At the right side of the figure, molecular weight markers have been indicated, respectively from top to bottom: 23.1, 9.4, 6.7, 4.4., 2.3. 2.0 and 0.6 kilobase pairs. The arrow indicates the position of the internal T-DNA fragment, containing the chimaeric kanamycin resistance gene present in the transformants but absent in the control lane. The other hybridizing bands are T-DNA/plant DNA fusion fragments.

progeny into both kanamycin resistant and sensitive plants in Mendelian ratios, e.g. in the selfed progeny of ATW7, 129 kanamycin resistant and 39 kanamycin sensitive plants were observed and for ATW15 this ratio was 83:25, which data fit a 3:1 ratio for the segregation of kanamycin resistance as a monogenic dominant trait.

Table II. The number of microcalli and kanamycin resistant transformants following treatment of MsK93 protoplasts with various plasmids. In Expt. 1, 3×10^5 protoplasts and in Expt. 2, 5×10^5 protoplasts were used per treatment.

Expt.	Plasmid used	Number ($\times 10^3$) of microcalli tested for kanamycin resistance	No. of transformants
1	pCTW19	1.0	0
	pCTW22	2.3	2
2	neoΔ18	4.1	0
	pCTW22	8.2	1
	pCTW78	7.2	1
Total		20.8	4

Direct gene transfer

When protoplasts, derived from callus cultures, were treated with plasmids containing the kanamycin resistance gene, kanamycin resistant calli were obtained at a frequency of about 5×10^{-4} (Table II). The number of transformants and the limited number of experiments did not allow the detection of statistical differences in the frequencies of transformation between the different plasmids that were used. Thus far, rooted plants have been obtained only from the pCTW 78-transformant (TMJ 78-1). All plants regenerated from this transformant showed an abnormal morphology and high, aneuploid chromosome numbers (56, 64, 70 and 76 for the 4 plants analysed). Southern blot analysis of callus DNA isolated from 4 different transformants confirmed the presence of at least one intact chimaeric kanamycin resistance gene (Fig. 3).

Discussion

The difference between *L. esculentum* and *L. peruvianum* in their ability to regenerate plants from cell and protoplast cultures is likely to be a quantitative difference. Thus, it has been shown recently by Shahin [23] that plants could be regenerated from protoplasts of several *L. esculentum*

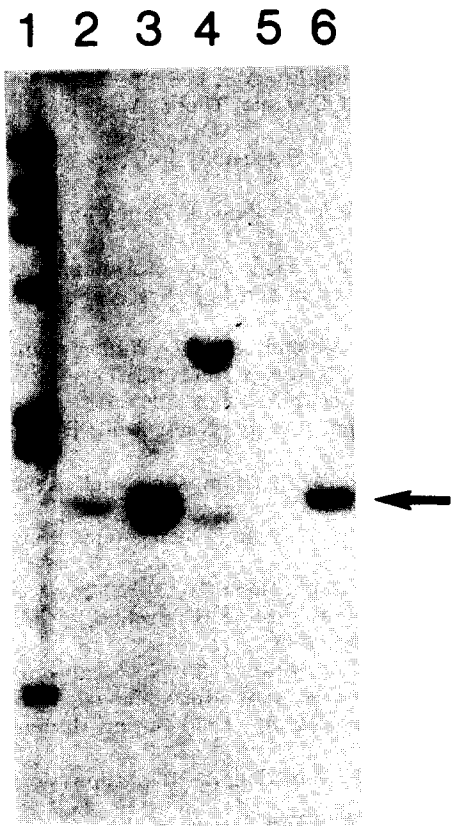

Fig. 3. Southern blot analysis of an EcoRI/HindIII double digest of DNA isolated from callus tissue of MsK93 direct gene transfer transformants (TMJ numbers). Lane 1, phage lambda DNA × HindIII; lane 2, TMJ 22-2; lane 3, TMJ 22-3; lane 4, TMJ 78-1; lane 5, the untransformed control MsK93; lane 6 reconstruction of 2 copies of the kanamycin resistance gene per tomato genome. The 1.4 kilobase pair EcoRI-HindIII fragment hybridizing in lanes 2, 3, 4 and 6 (see arrow) indicates the presence of an intact chimaeric kanamycin resistant gene. The other bands correspond to additional segments of the kanamycin gene fused to plant DNA.

cultivars, provided a tight regime of pre-treatment conditions was applied to the plant material prior to protoplast isolation. Nevertheless, despite these useful technical improvements in culture conditions, tomato is still considered a rather recalcitrant species in many laboratories. Thus, a new *L. esculentum* genotype which can be routinely regenerated from cell and protoplast cultures would be desirable in both fundamental and applied tomato research. By introducing the favourable regeneration characteristics from *L. peruvianum* into *L. esculentum* by conventional breeding, we have raised a genotype which is amenable to cell culture and genetic manipulation. The relatively large proportion of plants in the progeny of MsK93 which can be regenerated from established callus cultures and the apparent dominance of the regeneration capacity are characters which should facilitate the introduction of this trait into *L. esculentum* upon further backcrossing. (A detailed genetic analysis will be published elsewhere.) The ability of MsK93 to generate much more shoots per explant as compared with the tomato cultivars currently used, renders this new genotype highly attractive for introducing foreign genes into tomato using manipulated *A. tumefaciens* strains and the leaf disc transformation procedure of Horsch et al. [13]. The relatively short period of tissue culture required to obtain rooted transformants should reduce the number of transformants with aberrant chromosome numbers.

We have shown for the first time that kanamycin resistant tomato plants can be obtained following treatment with chimaeric plasmids. Direct gene transfer to protoplasts as compared to *A. tumefaciens*-mediated gene transfer may be of interest for studying transient gene expression and the mechanism of DNA integration. For this type of research, the non-diploid and aneuploid chromosome numbers of tissues and plants derived from established callus cultures should be less relevant. Recently, conditions have been established for electrically introducing DNA into dicot and monocot cells at a high efficiency [24]. Application of this electroporation technique to MsK93 protoplasts should greatly facilitate direct gene transfer in tomato.

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